AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph at page 7, lines 3-8 as follows.

Figure 6 is a ribbons representation of IκB-α (1ikn). DVNA (208-211) motif (note the beta-bulge) displayed as stick with H-bonds (dashed lines) from Asn210 (N210) side-chain to the backbone atoms of repeat 5. Modeling a hydroxyl group on Asn210 Cβ, in the same stereochemistry (*pro-S*) as is found for hydroxylated HIF-1α, shows how the creation of a H-bond between the side chain of Asp208 (D208) and the introduced hydroxyl of Asn210 may stabilize the beta-bulge. Residues Gly240 (G240) and Asp242 (D242) are also shown. The numbers 2.7, 2.8, and 3.3 are the lengths of the hydrogen bonds in Angstroms.

Please amend the paragraph at page 38, lines 14-24 as follows.

HeLa and U2OS cell extracts were prepared in Igepal lysis buffer (100 mM NaCl, 20 mM Tris –HCl pH 7.6, 5 mM $MgCl_2$ 0.5% Igepal CA630 containing "Complete" protease inhibitor, Roche Molecular Biochemicals). To analyse the p105/FIH interaction, p105 immunoprecipitations were performed using an antibody directed against the C-terminal region of p105 (Salmeron *et al.*, J. Biol. Chem. (2001) **276** 22215-22222). Following SDS-PAGE, immunoprecipitated proteins were transferred on to IMMOBILON™ ~~Immobilon~~-P membrane (Millipore) and processed for immunoblotting with a monoclonal anti-FIH antibody (prepared in host laboratory). To analyse the IkBα/FIH interaction, FIH immunoprecipitations were performed using polyclonal FIH antiserum (prepared in host laboratory) and immunoblotted with anti-IkBα antibody (clone 10b, Prof R. Hay, St. Andrew's University).

Please amend the paragraph on page 38, line 27 through page 39, line 7 as follows.

Plasmid corresponding to the ankyrin repeat domain (ARD) of p105, (amino acid residues 537-809) downstream of glutathione S-transferase (GST) (Bell *et al.* Mol.Cell.Biol. (1996) 16 6477-6485) was transformed into *E.coli* BL21(DE3) and grown at 37°C in 2TY medium supplemented with 100ug/ml ampicillin. Once the OD_{600} reached 0.8 the temperature was reduced to 28°C and IPTG was added to 0.5 mM. Cells were harvested 4hrs later by

centrifugation. The GST-tagged protein was purified using standard protocols with Glutathione SEPHAROSE™ ~~SepharoseTM~~ 4B resin (Amersham Biosciences). The tag was cleaved where necessary using thrombin. A second purification step using the Glutathione SEPHAROSE™ ~~SepharoseTM~~ 4B resin yielded protein of >90% purity by SDS-PAGE analysis. The GST p105 L668K mutant was made using the Quickchange system (Stratagene) and the following primers (forward: GCCTGCCATGTTTGAAGCTGCTGGTGGCCGC (SEQ ID NO:6) and reverse: GCGGCCACCAGCAGCTTCAAACATGGCAGGC (SEQ ID NO:7)).

Please amend the table beginning at page 39, line 17 to include a third new column as follows.

Name	Sequence	SEQ ID NO.
p105	SLPCLLLLVAAGADV**N**AQEQK	2
IkappB-alpha	YLGIVELLVSLGADV**N**AQEPC	1
FEM-1	NALVTKLLLDCGAEV**N**AVDNE	3
CAD	DESGLPQLTSYDCEV**N**API	11
Bcl-3	SLSMVQLLLQHGANV**N**AQMY	12
P19-INK4d	FLDTLKVLVEHGADV**N**VPDG	13
GABPbeta	HASIVEVLLKHGADV**N**AKDM	14
Tankyrase-1/2	NLEVAEYLLEHGADV**N**AQDK	15
2-5A-d-R	VEVLKILLDEMGADV**N**ACDN	16
Gankyrin/p28-II	RDEIVKALLGKGAQV**N**AVNQ	17
Myotrophin	QLEILEFLLLKGADI**N**APDK	18
M110	YTEVLKLLIQAGYDV**N**IKDY	19
FGIF	NTRVASFLLQHDADI**N**AQTK	20

Please amend the paragraph at page 40, lines 8-22 as follows.

For expression in 293T cells, pcDNA 3.1/GS plasmid containing p105 with a C-terminal PK-epitope tag (Invitrogen) was modified using the Quickchange system (Stratagene). The L668K mutation was made using primers described earlier. The R870A mutation with primers (forward: TCTGGGGGTACAGTCGCAGAGCTGGTGGAGGC, SEQ ID NO:8) and (reverse: GCCTCCACCAGCACTGCGACTGTACCCCCAGA, SEQ ID NO:9). The resulting p105 L668K/R870A plasmid was transiently transfected into 293T cells (5 μg DNA / 15 cm plate) using FUGENE™ ~~Fugene~~ 6 (Roche Molecular Biochemicals). 36h post-transfection, cells were lysed in 2 ml (100mM NaCl, 0.5 % Igepal CA630, 20mM Tris-HCl pH7.6, 5mM $MgCl_2$, containing "Complete" protease inhibitor (Roche Molecular Biochemicals) / 15 cm plate. Lysates were then centrifuged at 10,000 x g for 15 min at 4 °C. The lysate was then incubated with 30 μl anti-PK agarose conjugate (anti-V5, clone V5-10 Agarose conjugate, Sigma) on a rotator for 90 min at 4 °C. Beads were then washed 6 times in lysis buffer and purified protein resolved by SDS-PAGE.

Please amend the paragraph on page 41, lines 2-7 as follows.

Both *in vitro* and *in vivo* produced p105 samples were analysed using an Ettan™ MALDI-ToF Pro mass spectrometer in reflectron mode (Amersham Biosciences) with ANG III and hACTH peptides as internal standards. Tryptic fragments of *in vitro* produced IkappaB-alpha were analysed using a MALDI-TOF 2T mass spectrometer (MICROMASS™ ~~Micromass~~) in reflectron mode using Substance P, hACTH and insulin (B chain, oxidized) calibration standards.

Please amend the paragraph on page 41, lines 10-14 as follows.

A Jupiter C4 HPLC column (15cm × 4.6mm) was used to purify the substrate peptides following incubation with FIH using a linear gradient of 5-95% acetonitrile/0.05% formic acid at a flow rate of 1ml/min. The eluate from the column was analysed by a MICROMASS™ ~~Micromass~~ ZMD quadrupole mass spectrometer in positive mode.

Please amend the paragraph on page 45, lines 24-31 as follows.

To demonstrate *in vivo* hydroxylation of p105, cells were transfected with a full length p105 L668K/R870A mutant. A second mutation was introduced to remove the production of a peptide of mass 1,542 Da (not present in p105 ARD) that would overlap with the expected hydroxylated peptide. Tryptic digestion or protein recovered from gel purification of p105 L668K/R870A was carried out and the sample analysed by LC-MS using electrospary ionization MS/MS. MS/MS analyses identified the correct sequence of the expected peptide (VAAGADVNAQE, SEQ ID NO:4), and demonstrated the position of hydroxylation to be Asn778 (Figure 4).

Please amend the paragraph at page 48, line 21 through page 49, line 2 as follows.

A 5 litre suspension of HeLa S3 cells grown in DMEM was pelleted by centrifugation and resuspended in 250ml of Igepal 16-30 lysis buffer (Sigma). The cell lysate was cleared by centrifugation at 20,000g for 20 minutes. 25ml of protein A-SEPHAROSE™ ~~Sepharose~~ bead conjugates were first added to the cell lysate and incubated overnight at 4°C to precipitate proteins that non-specifically interacted with the SEPHAROSE™ ~~Sepharose~~ beads. IKB-α 10B antibodies were conjugated to SEPHAROSE™ ~~Sepharose~~ beads and incubated with the cell lysate for 8 hours with rotation at 4°C. The supernatant was removed and retained for analysis by SDS-PAGE/Western blot. The antibody-SEPHAROSE™ ~~Sepharose~~ bead conjugates were washed 7 times with 50 volumes of lysis buffer, to remove proteins non-specifically associated with the conjugates. IKB-α was eluted from the antibodies using 4ml 10mM glycine, pH 2.5 and concentrated by precipitation with 10% trichloroacetic acid. The precipitate was washed with 100µl of cold acetone, resuspended in 100µl of 1X SDS-PAGE loading buffer and subjected to SDS-PAGE analysis.

Please amend the paragraph at page 49, lines 5-14 as follows.

IKB-α was obtained via immunoprecipitation described in (a). Bands corresponding to IKBA were excised from the gel, cut into ~1mm squares and washed extensively with water. The

gel pieces were then washed with 100µl 50% acetonitrile for 15 minutes with shaking. After removing the supernatant, the gel pieces were incubated in 50µl acetonitrile at room temperature without shaking, for. After 5 minutes, excess acetonitrile was removed and the gel pieces were rehydrated in 50µl 0.1M ammonium bicarbonate (Sigma). After a further 5 minutes, 50µl acetonitrile was added and the mixture incubated at room temperature without shaking. After 15 minutes, all excess liquid was removed and the gel pieces were dried in a SPEEDVAC™ ~~SpeedVac~~.

Please amend the paragraph at page 49, lines 15-21 as follows.

The dried gel pieces were rehydrated in 50µl 10mM DTT (dissolved in 0.1M ammonium bicarbonate) and incubated at 56°C for 45 minutes. Excess DTT was removed, 50µl of 55mM iodoacetamide (dissolved in 0.1M ammonium bicarbonate) added and incubated at room temperature for 30'. All excess liquid was removed and the gel pieces were washed again with ammonium bicarbonate/acetonitrile as described above. The gel pieces were dried in a SPEEDVAC™ ~~SpeedVac~~ and resuspended in 50µl of 20µl/ml porcine trypsin (Promega) and incubated overnight at 37°C.

Please amend the paragraph at page 49, lines 22-31 as follows.

After incubation, the supernatant was transferred to a clean tube and the gel pieces were washed with 100µl 25mM ammonium bicarbonate for 5 minutes, with shaking. An additional 100µl acetonitrile was added and left shaking for a further 60 minutes. The supernatant was removed and retained. The gel pieces were washed with 100µl 0.1% trifluoroacetic acid (Fischer) for 5 minutes, with shaking. An additional 100µl acetonitrile was added and left shaking for a further 60 minutes. All supernatants were pooled and dried in a SPEEDVAC™ ~~SpeedVac~~. The pellet was resuspended in 20µl 0.1% trifluoroacetic acid and incubated overnight at 50°C. All liquid was dried in a SPEEDVAC™ ~~SpeedVac~~ and resuspended in 5% acetonitrile/0.1% formic acid, prior to mass spectrometry.

Please amend the paragraph at page 50, lines 2-9 as follows.

Liquid Chromatography/Mass spectrometry (LC/MS) was performed using a C4 Jupiter HPLC column (Phenomenex). Peptides were separated using a linear gradient of 5-95% acetonitrile/0.1% formic acid and analysed using a Waters Q-TOF™ ~~Q-Tof~~ MICROMASS™ ~~Micro Mass~~ Spectrometer. Hydroxylated IKB-α eluted from the column after 8.5 minutes and the identity of the peptide (CGADVN*R (SEQ ID NO:23, with N defined as follows) was confirmed by tandem mass spectrometry (MS/MS) where N* corresponds to the hydroxylated asparagine residue. (The N-terminal cysteine was modified by alkylation and susbequent dehydration).